Bull. Fac. Agr., Saga Univ. No. 85 : 109～116 (2000)

Action Pattern of Chitosanase from *Bacillus amyloliquefaciens* UTK on Partially *N*-Acetylated Chitosan

Kiyohiko SEKI, Koji TATEISHI and Yasushi UCHIDA
(Laboratory of Food Resource Chemistry)
Received September 11, 2000

Summary

The hydrolysates of partially *N*-acetylated chitosan by chitosanase from *Bacillus amyloliquefaciens* UTK (UTK chitosanase) were separated using CM-Sephadex C-25 column chromatography, gel filtration on Bio-Gel P-2, and HPLC on μ-Bondapak NH_2. Sugar sequences of the oligosaccharides were identified by exo-splitting with β-*N*-Acetylglucosaminidase and exo-β-glucosaminidase, and by MALDI-TOF MS. In addition to chitooligosaccharides, $(GlcN)_{2\text{-}4}$, hetero-chitooligosaccharides such as $(GlcN)_2$-GlcNAc, $(GlcN)_2$-GlcNAc-$(GlcN)_2$, $(GlcN)_2$-GlcNAc-$(GlcN)_2$, and $(GlcN)_2$-GlcNAc-$(GlcN)_2$-GlcNAc were detected. Since these oligosaccharides had GlcN at the non-reducing ends and GlcN or GlcNAc at the reducing ends, it was evident that the enzyme can cleave GlcN-GlcN and GlcNAc-GlcN linkages. UTK chitosanase is classified into subclass I.

Key words : chitosanase, *Bacillus amyloliquefaciens* UTK, family 46, subclass I, action pattern,

Introduction

Chitosanase has been generally recognized as an enzyme that attack chitosan but not chitin. The Enzyme Commission defines chitosanase (EC 3.2.1.123) as an enzyme that catalyzes the endo hydrolysis of β-1,4-glycosidic linkages between *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN) residues in a partially *N*-acetylated chitosan. The Enzyme Commission definition of chitosanase is accompanied by a comment that chitosanase "acts only on polymers with 30% to 60% acetylation." The definition of this enzyme no longer holds, according to more recent findings on chitosanase. From the viewpoint of enzyme definition alone, further information about the structure and function of chitosanase is needed.

Most chitosanases are found in microorganisms[1-6], and a few are found in plants[7]. *Bacillus amyloliquefaciens* UTK, which was isolated from soil, was found to produce constructively most potent extracellular chitosanase. This chitosanase belongs to the family 46 of glycoside hydrolases. In this paper, we describe how the UTK chitosanase specifically hydrolyzes the β-glycosidic linkages in partially *N*-acetylated chitosan.

Materials and Methods

Materials. Chitosan with 28% *N*-acetylation, which was prepared by homogeneous alka-

line hydrolysis of chitin, was the product of Katakura Chikkarin Co., Ltd.. Bio-Gel P-2 was obtained from Bio-Rad Laboratories. A series of $(GlcN)_n$ (n=2-6) was purchased from Seikagaku Kogyo Co., Ltd.. Other reagents were of analytical grade.

Chitosanase was purified from the culture broth of *Bacillus amyloliquefaciens* UTK. The specific activity of the enzyme was 800 units per mg protein. Chitosanase activity was assayed by soluble chitosan as a substrate[8]. Exo-β-glucosaminidase (β-GlcNase) was prepared from *Penicillium* sp. AF9-P-128 as described previously[9]. β-*N*-Acetylglucosaminidase (β-GlcNAcase) from *Pycnopourus cinnabarinus* IFO 6139 was a gift from Prof. M. Mitsutomi of Saga University. β-GlcNase and β-GlcNAcase activities were assayed by the method of Mitsutomi *et al.*[10]

Measurement of reducing sugar. The amount of reducing sugar in column chromatograpies was measured by a modification of Schales method with GlcN standard[11].

Hydrolysis of chitosan by chitosanase. The hydrolysis of chitosan was done as follows. A reaction mixture consisting of 0.6% chitosan (28% *N*-acetylated chitosan) in 500 ml of 0.1 M acetate buffer (pH 4.7), 5 ml of 2% NaN_3, and 1.5 ml of chitosanase (36 units) was incubated for 24 hours at 30℃. Then, after the addition of another 1.5 ml of chitosanase (36 units), the mixture was further incubated for 24 hours at 30℃. The reaction was stopped by boiling for 5 minutes. The reaction mixture was concentrated in a rotary evaporator under reduced pressure at a temperature below 35℃ and dialyzed through an electric dialyzer (Micro Acilyzer G1, Asahikasei Kogyo Co., Ltd.). The dialyzed solution was adjusted to pH 4.5 by adding acetic acid.

Separation of oligosaccharides. The dialyzed hydrolysate was put onto a column (2.6 x 53 cm) of CM-Sephadex C-25 previously equilibrated with 0.02 M acetate buffer (pH 4.5). After the column was washed with the same buffer, oligosaccharides were eluted with a linear gradient from 0 to 1.3 M NaCl in the same buffer, at a flow rate of 30 ml per hour. The dialyzed and concentrated oligosaccharides were put on a column (2.6 x 180 cm) of Bio-Gel P-2 previously equilibrated with 0.15 M acetate buffer (pH 4.2) and eluted with the same buffer at a flow rate of 15 ml per hour.

High performance liquid chromatography. The HPLC system consisted of an 880-PU pump, an 830-RI detector (Japan Spectroscopic Co., Ltd.), and a D-2500 Chromato-Integrator (Hitachi Ltd.). Sugars were separated on a Radial-PAK μ-Bondapak NH_2 column (8.0 x 100 mm, Waters Associates) using acetonitrile-water mixture (65 : 35, v/v) as the mobile phase, at a flow rate of 2.0 ml per minute. Chitooligosaccharides were detected by monitoring the refractive index.

Mass spectrometry. Mass spectral analysis of oligosaccharides was done by a Voyager Elite matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF MS) (PerSeptive Biosystems). The acceleration voltage was 20 kV and 2,5-dehydroxybenzoic acid used as the matrix.

Exoglycosidase digestion. Partially *N*-acetylated chitooligosaccharides were hydrolyzed by successive action of β-GlcNase and GlcNAcase, and the hydrolysis products were analyzed by HPLC.

Results and discussion

To clarify the specificity for hydrolyzing linkages of recombinant chitosanase, structures of oligosaccharides generated from partially *N*-acethylated chitosan by the action of the enzyme were studied. Chitosan with 28% *N*-acetylation was treated with the enzyme and the hydrolysate was separated by CM-Sephadex C-25 column chromatography (Fig. 1). Although oligosaccharides consisting of only GlcNAc are eluted with 0.02 M acetate buffer (pH 4.5), no reducing sugar was eluted with the same buffer indicating that the hydrolysate did not contain $(GlcNAc)_n$. Each fraction was collected, dialyzed through an electric dialyzer, and lyophilized. It was further purified by gel filtration on a Bio-Gel P-2 column. The hydrolysate was separated into eight peaks. Fractions F-1, 2, 4, 5, 6, 7, and 8 were homogeneous based on the HPLC. Therefore, they were used for sugar composition and sequence analyses.

The structures of chitooligosaccharides were studied by exoglycosidase digestion and MALDI-TOF MS. In MALDI-TOF MS, pseudo molecular ions of chitooligosaccharides were detected in the positive ion mode as sodium adducts. In the mass spectra of F-2, 4, and 7, the ion peaks at m/z 363.0 $[(GlcN+Na)^+]$, 524.0 $[[(GlcN)_2+Na]^+]$ and 685.1 $[[(GlcN)_3+Na]^+]$ were observed, respectively. These oligosaccharides were completely decomposed by β-GlcNase to only

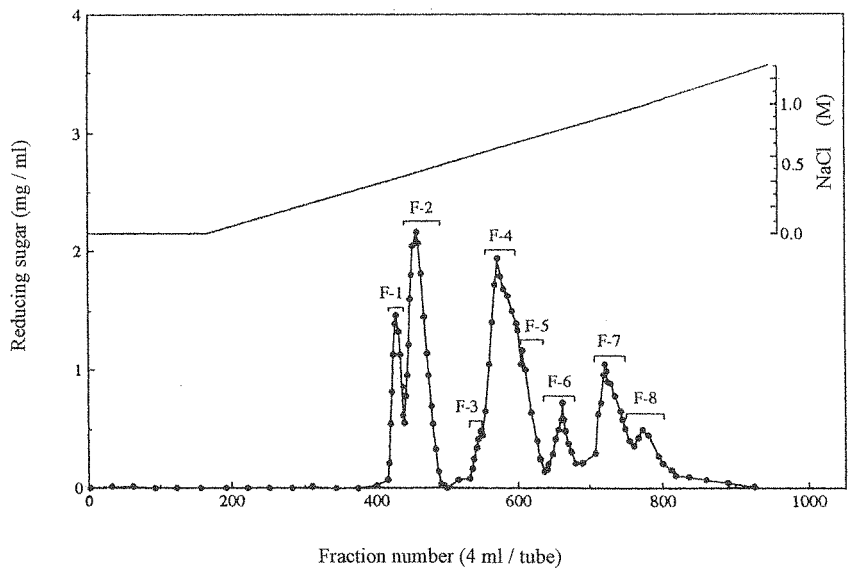


Fig. 1 CM-Sephadex C-25 Column Chromatography of Oligosaccharides Produced in the Hydrolysis of Partially *N*-Acetylated Chitosan with Chitosanase from *Bacillus amyloliquefaciens* UTK.

The hydrolysate from 3g of *N*-acetylated chitosan was put onto a column of CM-Sephadex C-25. The experimental details are described in the text. Oligosaccharides were eluted with a linear gradient from 0 to 1.3 M NaCl in the 0.02 M acetate buffer (pH 4.5) ●, reducing sugar, Line : -, NaCl.

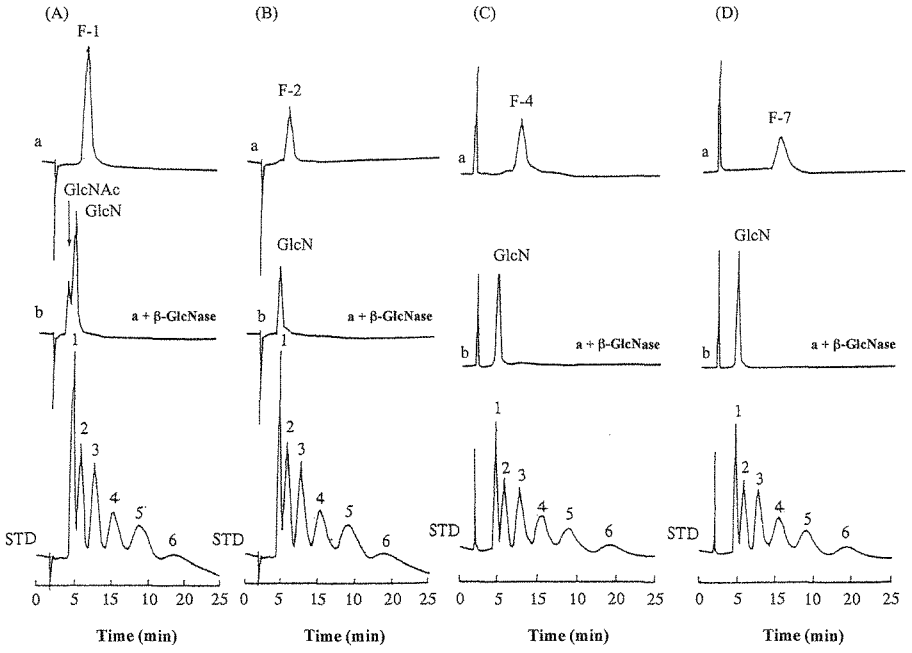


Fig. 2 HPLC of the Hydrolysis Products of F-1 (A), F-2 (B), F-4 (C), and F-7 (D) by β-GlcNase.

The experimental details are described in the text. Hydrolysis products were eluted with acetonitrile-water (65 : 35, v/v) and monitored by the refractive index. Chitooligosaccharide (a) was digested with β-GlcNase (b).

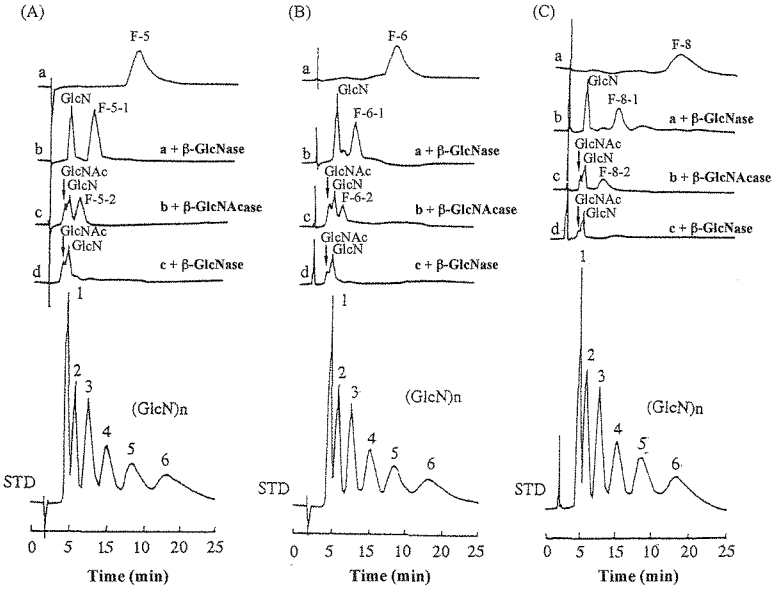


Fig. 3 HPLC of the Hydrolysis Products of F-5 (A), F-6 (B), and F-8 (C) by Successive Action of β-GlcNase and β-GlcNAcase.

The experimental details are described in the text. Hydrolysis products were eluted with acetonitrile-water (65 : 35, v/v) and monitored by the refractive index. Chitooligosaccharide (a) was digested with β-GlcNase (b) followed by β-GlcNAcase (c) and β-GlcNase (d).

GlcN (Fig. 2). Thus, F-2, 4, and 7 were identified to be $(GlcN)_2$, $(GlcN)_3$, and $(GlcN)_4$, respectively.

In the MALDI-TOF MS analysis of F-1, 5, 6, and 8, the $[M+Na]^+$ ions were detected at m/z 566.1, 1091.3, 888.2, and 1049.4, indicating that mother compounds were monoacetylchitotriose, diacetylchitohexaose, monoacetylchitopentaose, and monoacetylchitohexaose, respectively. To determine the sugar sequences of these hetero-chitooligosaccharides, the samples were hydrolyzed by successive action of β-GlcNase and β-GlcNAcase. Figure 2 and 3 show the HPLC pattern of the hydrolysates of each sample.

F-1, which was monoacetylchitotriose, was hydrolyzed to GlcN and GlcNAc by β-GlcNase. From these results, F-1 was identified to be $(GlcN)_2$-(GlcNAc).

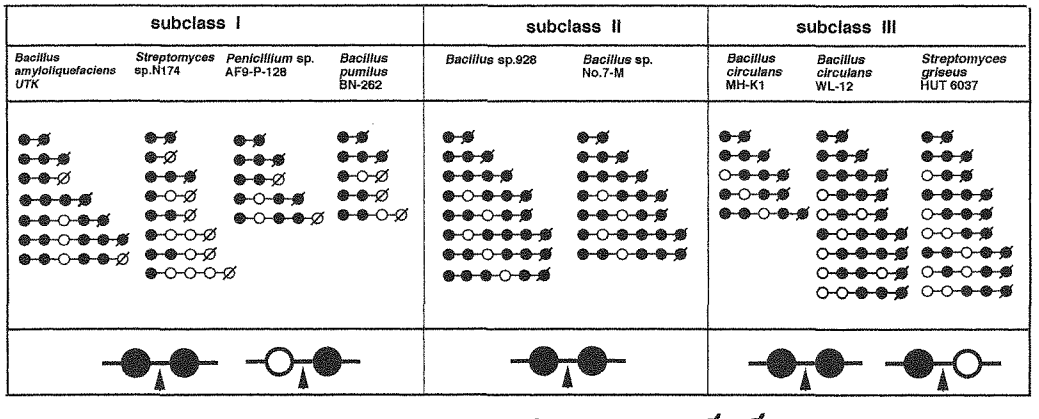
F-5 was hydrolyzed by β-GlcNase to produce GlcN and F-5-1, and the later was further split by β-GlcNAcase to produce GlcNAc and F-5-2. MALDI-TOF MS analysis indicated that F-5-1 and F-5-2 were tetrasaccharides containing two GlcNAc residues [$(M+Na)^+$; m/z 769.0] and trisaccharides containing one GlcNAc residue [$(M+Na)^+$; m/z 566.1], respectively. F-5-2 was hydrolyzed to GlcN and GlcNAc by β-GlcNase. From these results, F-5 was identified as $(GlcN)_2$-(GlcNAc)-$(GlcN)_2$-(GlcNAc).

F-6, which was monoacetylchitopentaose, was hydrolyzed by β-GlcNase to GlcN and F-6-1. MS analysis revealed that F-6-1 was monoacetylchitotriose [$(M+Na)^+$, m/z 566.1]. These results indicated that the structure of F-6 was $(GlcN)_2$-(GlcNAc)-$(GlcN)_2$.

F-8, which was monoacetylchitohexaose, was hydrolyzed by β-GlcNase to GlcN and F-8-1. In the mass spectrum of F-8-1,the $[M+Na]^+$ ion was observed at m/z 727.2 which corresponded to (monoacetylchitotriose+Na)$^+$. These results indicated that the structure of F-8 was $(GlcN)_2$-(GlcNAc)-$(GlcN)_3$.

From the results described above, it can be concluded that this chitosanase hydrolyze GlcN-GlcN and GlcNAc-GlcN linkages but not GlcN-GlcNAc nor GlcNAc-GlcNAc linkages in partially *N*-acetylated chitosan.

Table 1. Oligosaccharides obtained in the digestion of partially *N*-acetylated chitosan by chitosanases



Chitosanases are classified on the basis of amino acid sequence similarities into families 8, 46, 75, and 80 among the 81 glycosyl hydrolase families[12]. Family 46 chitosanase is further subdivided into some groups, such as N174 chitosanase, UTK chitosanase, and MH-K1 chitosanase. On the other hand, microbial chitosanases are classified into other three subclasses based on the specificity of the cleavage positions for partially acetylated chitosan[4] (Table 1). Subclass I chitosanases such as N174 chitosanase[13] and UTK chitosanase, can split GlcN-GlcN and GlcNAc-GlcN linkages, whereas *Bacillus* sp. No.7-M chitosanase[3] in subclass II can cleave only GlcN-GlcN linkage. Subclass III chitosanase such as MH-K1 chitosanase[10] can split both GlcN-GlcN and GlcN-GlcNAc linkages. This selectivity at the cleavage position of the substrates might be controlled by rigid substrate recognition by chitosanases in these subclasses.

Acknowledgements

We are grateful to Prof. Masaru Mitsutomi of Saga University for his generous gift of β-GlaNAcase and to Dr. Yuto Kamei and Dr. Hideaki Harada in Marine and Highland Bioscience Center, Saga University for help in mass spectral analysis.

References

1. Sakai, K., R. Katsumi, A. Isobe, and F. Nanjo (1991). Purification and hydrolytic action of a chitosanase from *Nocardia orientalis*. *Biochim Biophys Acta.* 1079, 65-72.
2. Boucher, I., A. Dupuy, P. Vidal, W. A. Neugebauer, and R. Brzezinski (1992). Purification and characterization of chitosanase from *Streptomyces* N174. *Appl. Microbiol. Biotechnol.* 38, 188-193
3. Izume, M., S. Nagae, H. Kawagishi, M. Mitsutomi, and A. Ohtakara (1992). Action pattern of *Bacillus* sp. No.7-M chitosanase on partially *N*-acetylated chitosan. *Biosci. Biotechnol. Biochem.* 56, 448-453
4. Fukamizo, T., T. Ohkawa, Y. Ikeda, and S. Goto (1994). Specificity of chitosanase from *Bacillus pumilus*. *Biochim. Biophys. Acta* 1205, 183-188
5. Seki, K., Kuriyama H, Okuda T, and Uchida Y (1997). Molecular cloning oh the gene encoding chitosanase from *Bacillus amyloliquefaciens* UTK. *Advances in Chitin Science* 2, 284-289
6. Park, J. K., K. Shimono, N. Ochiai, K. Shigeru, M. Kurita, Y. Ohta, K. Tanaka, H. Matsuda, and M. Kawamukai (1999). Purification, characterization, and gene analysis of a chitosanase (ChoA) from *Matsuebacter chitosanotabidus* 3001. *J Bacteriol.* 181, 6642-9.
7. Osswald, W. F., J. P. Shapiro, H. Doostdar, R. E. McDonald, R. P. Niedz, C. J. Nairn, C. J. Hearn, and R. T. Mayer (1994). Identification and characterization of acidic hydrolases with chitinase and chitosanase activities from sweet orange callus tissue. *Plant Cell Physiol.* 35, 811-20.
8. Mitsutomi, M., M. Isono, A. Uchiyama, and T. Watanabe (1998). Chitosanase activity of the enzyme previously reported as β-1, 3-1, 4-glucanase from *Bacillus circulans* WL-12. *Biosci. Biotechnol. Biochem.* 62, 2107-2114
9. Uchida, Y., H. Takeda, A. Ohkuma, and K. Seki (1997). Purification and properties of exo-β-D-glucosaminidase from *Penicillium* sp., and its applications. *Advances in chitin Science* 2, 244-249
10. Mitsutomi, M., M. Ueda, M. Arai, A. Ando, and T. Watanabe (1996). Action patterns of microbial chitinases and chitosanases on partially *N*-acetylated chitosan. *Chitin Enzymol.* 2, 273-284
11. Imoto, T. and K. Yagishita (1971). A simple activity measurement of lysozyme. *Agric. Biol. Chem.* 35, 1154-1156
12. Henrissat, B. and A. Bairoch (1996). Updating the sequence-based classification of glycosyl hydrolases. *Bio-*

chem. J. 316, 695-696

13. Fukamizo, T., Y. Honda, S. Goto, I. Boucher, and R. Brzezinski (1995). Reaction mechanism of chitosanase from *Streptomyces* sp. N174. *Biochem. J.* 311, 377-383

Bacillus amyloliquefaciens UTK が生産するキトサナーゼの部分 *N* アセチルキトサンに対する作用機作

関　清彦・立石　浩二・内田　泰
(生物資源化学研究室)
平成12年9月11日　受理

摘　要

キトサナーゼは，アミノ酸の疎水性クラスター分析による分類から，ファミリー46を代表とする，4種のファミリーに属している．また，一方部分 *N*-アセチルキトサンに対する作用様式から，3種に分類されており，構造と機能の相関について興味が持たれている．我々は，土壌中より単離された *Bacillus amyloliquefaciens* UTK が構成的に活性の強いキトサナーゼを生産することを見出し，この酵素がファミリー46に属するキトサナーゼであることを明らかにしてきた．本研究ではこの酵素の分解様式の解明を試みた．本酵素による，*N*-アセチルキトサンの分解生成物を CM-Sephadex C-25イオン交換クロマトグラフィー，Bio-Gel P-2によるゲルろ過，μ-Bondapak NH_2カラムを用いた HPLC により分離した．分解生成物は主に8の画分からなっていた．各画分の糖配列は，エキソグルコサミニダーゼおよびエキソ *N*-アセチルグルコサミニダーゼによる分解，MALDI-TOF MS による質量分析により $(GlcN)_{2\text{-}4}$, $(GlcN)_2$-GlcNAc, $(GlcN)_2$-GlcNAc-$(GlcN)_2$, $(GlcN)_2$-GlcNAc-$(GlcN)_2$, $(GlcN)_2$-GlcNAc-$(GlcN)_2$-GlcNAc であると決定された．これら，分解生成物は非還元末端が GlcN であり，還元末端は GlcN あるいは GlcNAc であったことから，GlcN-GlcN 結合と GlcNAc-GlcN の結合を切断しうることが証明された．このことから，ファミリー46キトサナーゼである本酵素は，サブクラスⅠの分解様式であることが明らかになった．